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Sample size matters in dietary gene expression studies—A case study in the gilthead sea bream (*Sparus aurata* L.)

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ABSTRACT

One of the main concerns in gene expression studies is the calculation of statistical significance which in most cases remains low due to limited sample size. Increasing biological replicates translates into more effective gains in power which, especially in nutritional experiments, is of great importance as individual variation of growth performance parameters and feed conversion is high. The present study investigates in the gilthead sea bream *Sparus aurata*, one of the most important Mediterranean aquaculture species. For 24 gilthead sea bream individuals (biological replicates) the effects of gradual substitution of fish meal by plant ingredients (0% (control), 25%, 50% and 75%) in the diets were studied by looking at expression levels of four immune-and stress-related genes in intestine, head kidney and liver. The present results showed that only the lowest substitution percentage is tolerated and that liver is the most sensitive tissue to detect gene expression variations in relation to fish meal substituted diets. Additionally the usage of three independent biological replicates were evaluated by calculating the averages of all possible triplets in order to assess the suitability of selected genes for stress indication as well as the impact of the experimental set up, thus in the present work the impact of FM substitution. Gene expression was altered depending of the selected biological triplicate. Only for two genes in liver (*hsp70* and *tgf*) significant differential expression was assured independently of the triplicates used. These results underlined the importance of choosing the adequate sample number especially when significant, but minor differences in gene expression levels are observed.

1. Introduction

For gene expression studies today, a wide range of methods is available. Each of the methods comprises advantages as well as disadvantages. For low throughput expression analysis the use of quantitative PCR (qPCR) has been widely applied and the technique has become an important technology in the area of functional genomics (Vandesompele et al., 2002). In order to obtain statistical significance in qPCR (i) PCR efficiency, (ii) selection of suitable reference genes as well as (iii) the number of biological replicates are of importance (Bustin, 2008). While in most qPCR publications the PCR efficiency as well as the selection of appropriate reference genes are investigated (e.g., De Santis et al., 2011), the number of required biological replicates are not discussed (Karlen et al., 2007; Kitchen et al., 2010). Statistical power in biological as well as in medical studies is, independently of the method used, of great importance (Button et al., 2013). Nevertheless in most cases statistical power remains low, due to low number of independent biological samples, which in the majority of gene expression studies is limited to three as increasing sample size leads to more cost and increased laboratory work. The use of three replicates means that the results have a large confidence range (confidence interval) and a low confidence level. Assuming for example a confidence level of 95% and a confidence interval of 10 the required sample size would be 96. Thus, increasing biological replicates translates into a more effective gain in power, which, especially in nutritional experiments, is of importance as individual variation of growth performance parameters and feed conversion is high (Jobling and Baardvik, 1994; McCarthy et al., 1992). Consequently, fine tuning of expression analysis may be of great importance especially for nutritional studies in aquaculture where fish welfare is highly dependent on adequate feed provision.

Protein requirements in teleost species depend on the availability of protein sources, their amino acid (AA) profile and dietary energy level. For carnivorous fish, fish meal (FM) is the main pro-

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tein source in aquaculture feeds and provides them with nitrogen required for the synthesis of non-essential AA (Bakke, 2011). However, due to the limited availability of FM as well as its high cost, FM replacement levels by plant proteins in feeds have increased steadily over the past years. On the other hand, the use of high levels of plant ingredients as FM alternatives have been proven to affect fish health in several fish species (Lilleeng et al., 2009; Olsen et al., 2007; Urán et al., 2008a,b; Kokou et al., 2012). First evidence of altered immune system in marine fish due to vegetable oils was published in Atlantic salmon (Bransden et al., 2003). Among potential substitutes soy products are the main alternatives added to the fish diet although that, at high plant protein (PP) concentrations in the diets, alterations in the immune system have been observed, as well as significant changes in intestinal morphology and immune-related gene expression levels (Baeverfjord and Krogdahl, 1996; Urán et al., 2008a,b). It is further known that the fish intestine is of importance not only for the absorption of nutrient and osmoregulation, but also for its role as immune tissue preventing certain pathogens to penetrate into the organism (Cain and Swan, 2010; Rombout Jan et al., 2011). Hence, the intestine and particularly the distal intestine is considered as one of the major tissues to investigate the effects of nutrition on the immune system (Baeverfjord and Krogdahl, 1996). For studying possible immune response another important tissue is the head kidney due to the central hematopoetic role (Rise et al., 2004). During the last decade also liver was recognized as a major organ of the immune system due to its key role as a mediator of systemic and local immunity presenting an important site for immune regulation (Castro et al., 2014).

The gilthead sea bream (*Sparus aurata* L.) along with the European sea bass (*Dicentrarchus labrax*) belongs to the main aquaculture species in the Mediterranean. The genomic toolbox of the gilthead sea bream has been significantly enriched in the recent years, facilitating the identification and characterization of genes. Furthermore high throughput studies have been performed in sea bream showing on the one hand that no transcriptome response occurred in the intestine after FM substitution and on the other hand that plant meal substitution influenced the intestine transcriptome after exposure to a bacterial pathogen (Bonaldo et al., 2008; Calduch-Giner et al., 2013; Montero et al., 2010, 2008). Yet, only little is known about how FM substitution affects gene expression of immune related genes. In this line, the present study selected as case study the effects of three diets on distal intestine, head kidney and liver of the gilthead sea bream. The diets comprised gradual fish meal substitution by plant ingredients (0% (control), 25%, 50% and 75%) in 24 individuals per diet. Expression levels of four immune-and stress-related genes i.e. heat shock protein 70 (*hsp70*), beta-2 microglobulin (*b2m*), transforming growth factor beta 1 (*tgf-β1*) and cathepsin S (*catS*) in all 24 biological replicates were assessed in head kidney, intestine and liver. In addition, to investigate the possible usage of only three independent biological replicates the averages of all possible triplets were calculated and the impact of the experimental set up was assessed, i.e. in the present work the impact of FM substitution.

2. Methods

2.1. Feeding trial: experimental diets and tissue sampling

Animal care was carried out according to the "Guidelines for the treatment of animals in behavioral research and teaching."

The experiment was held in triplicate groups of sea cages ($6 \times 6 \times 6$ m) in the fish farm of Platia (Saronic Gulf). In each cage 5.000 graded fish (A-class) were placed after hand-counted and weighed in the beginning and at the end of the experiment. Average initial weight of the fish was approximately 125 g. To estimate fish weight 10% of the population was weighed in the beginning, while in the end of the experiment fish were harvested and the total population was weighed. The duration of the trial was 236 days (9th September–4th May) and temperature, oxygen concentration and mortalities were recorded daily. Average water temperature was 18.9 °C and the range was 14.6–27.1 °C. Feeding rate was predicted by feeding tables according to the temperature and estimated biomass increase. The diets were produced by BioMar Hellenic S.A. (Volos, Greece) and labeled as "RH" (Research Hellas). Standard bream diet (Efico Sigma 463 4.5 mm) was used as the control diet (20% FM) and compared to three experimental diets with decreasing levels of fishmeal, a diet with 15% FM (RH30), a diet with 10% FM (RH31) and a diet with 5% FM (RH32). Thus, the experimental diets corresponded to 25% (RH30), 50% (RH31) and 75% (RH32) of FM substitution compared to the control diet. Main ingredients are shown in Table 1. The experimental period was 6 months and fish reached the size of 350 g prior to tissue sampling. At the end of the experiment out of 24 fish per dietary treatment (in total 96) the distal intestine, head kidney and liver were removed and stored in RNAlater at −80 °C (in total 288 samples) for gene expression studies.

Table 1
Diet composition of the experimental diets.

Ingredients (g/kg)	Feed code			
	Control	RH30	RH31	RH32
Fish meal (std. 70%)	200.00	140.00	90.00	40.00
Fish hydrolysate (CPSP 90)	10.00	10.00	10.00	10.00
Rapeseed oil	40.00	40.00	40.00	41.00
Soya cake (48%)	150.00	150.00	150.00	150.00
Soya protein concentrate (60%)	100.00	120.00	135.00	160.00
Corn gluten (60%)	162.00	196.00	235.00	265.00
Wheat	60.22	65.03	64.58	65.49
Sunflower cake (37%)	150.00	150.00	150.00	150.00
Fish oil std.	40.00	40.00	40.00	41.00
Fish oil trimmings (salmon)	79.00	80.00	81.00	81.00
Premix & additives	6.78	12.97	18.42	23.51
Nutrients				
Moisture (%)	9.14	7.77	6.87	5.69
Protein (%)	44.00	44.00	44.00	44.00
Fat (%)	19.00	18.82	18.57	18.40
Ash (%)	5.48	5.33	5.16	5.03
Cellulose (%)	4.21	4.32	4.40	4.54
Starch (%)	5.91	6.58	7.00	7.43
Digestible energy (MJ/kg)	17.13	17.15	17.12	17.14

RH = Research Hellas.

2.2. RNA extraction and reverse transcription

RNA was extracted using the Trizol protocol in combination with Lysing Matrix tubes D (Mp Biomedicals, Santa Ana, California, USA). RNA quantity was measured using Nanodrop Spectophotometer (NanoDrop Technologies Inc., Wilmington USA) and quality was assessed by agarose gel as well as by DNAnalyzer (Agilent, Santa Clara, California). 1 μg total RNA was reverse transcripted using reverse transcriptase (200 U/μl) (Invitrogen,/VWR, Tromsø, Norway). Dilutions of 1:50 for each of the 288 samples were used for further analysis.

2.3. Gene identification and primer design

Genes involved in immune response were selected based on current literature and their transcripts were retrieved out of the non-redundant (nr) and expressed sequence tags (est) database of NCBI (Table 2). Each sequence was evaluated using Blastx search of NCBI. Primer pairs for qPCR were designed using a combination of different software programs: NetPrimer, Primer3 and Beacon. All primers were standardized and tested by agarose gel and the melting temperature.

Table 2
Gene annotation.

Gene	Abbreviation	Accession #	Reference	Kegg pathway
Heat shock protein 70	HSP70	EU805481	Present study	ko04612 Antigen processing and presentation
Beta-2 microglobulin	B2M	FM148172	Present study	ko04612 Antigen processing and presentation
Transforming growth factor beta 1	*TGF-β1*	AF424703	Castillo et al. (2009)	*ko04010 MAPK signaling pathway* *ko04060 Cytokine-cytokine receptor interaction* *ko04110 Cell cycle* *ko04144 Endocytosis* *ko04350 TGF-beta signaling pathway* *ko04380 Osteoclast differentiation* *ko04672 Intestinal immune network for IgA production* *ko05140 Leishmaniasis* *ko05142 Chagas disease (American trypanosomiasis)* *ko05144 Malaria* *ko05145 Toxoplasmosis* *ko05146 Amoebiasis* *ko05152 Tuberculosis* *ko05166 HTLV-I infection* *ko05200 Pathways in cancer* *ko05210 Colorectal cancer* *ko05211 Renal cell carcinoma* *ko05212 Pancreatic cancer* *ko05220 Chronic myeloid leukemia* *ko05323 Rheumatoid arthritis* *ko05410 Hypertrophic cardiomyopathy (HCM)* *ko05414 Dilated cardiomyopathy*
Cathepsin S	*CatS*	EST database	Present study	ko04142 Lysosome ko04145 Phagosome ko04612 Antigen processing and presentation ko05152 Tuberculosis
Insulin growth factor 1	*IGF1*	EF563837.1	Perrot et al. (1999)	*ko04114 Oocyte meiosis* *ko04115 p53 signaling pathway* *ko04150 mTOR signaling pathway* *ko04510 Focal adhesion* *ko04730 Long-term depression* *ko04914 Progesterone-mediated oocyte maturation* *ko04960 Aldosterone-regulated sodium reabsorption* *ko05200 Pathways in cancer* *ko05202 Transcriptional misregulation in cancer* *ko05214 Glioma* *ko05215 Prostate cancer* *ko05218 Melanoma* *ko05410 Hypertrophic cardiomyopathy (HCM)* *ko05414 Dilated cardiomyopathy*

2.4. Quantitative PCR

In total five reference genes were tested out of which three were used for qPCR normalization. For qRT-PCR, DNA Engine Opticon 2 Continuous Fluorescence Detector (MJ Research, Massachusetts, USA) was used. Each reaction consisted of 5 μl of 50× diluted cDNA and 12.5 μl SYBR Green PCR Master Mix (Kapa SYBR Fast QPCR Kit, Woburn, MA, USA) and the final volume was adjusted to 25 μl with deionized Merck water. All samples were run in 2 technical replicates and 24 biological replicates. For each gene one reverse-transcription negative control was included. The reactions were performed according to the protocol of KAPA. In brief, after an initial denaturation step at 95 °C for 10 min, 40 amplification cycles of 15 s at 95 °C and 1 min at 57 °C (annealing and extension steps) followed. After each cycle a plate read for fluorescent signal assessment was performed. Finally a dissociation curve with a gradient of 50 °C to 95 °C was performed after thermo-cycling to evaluate the specificity of the amplification and to verify the absence of primer dimers.

2.5. Data acquisition

PCR efficiency as well as Ct-values were calculated based on the exported raw fluorescence data applying the Miner software (Zhao and Fernald, 2005). The Miner software is a method for quantifying qRT-PCR results using calculations based on the kinetics of individual PCR reactions without the need of standard curves (Zhao and Fernald, 2005). The raw fluorescence data fit as a function of PCR cycles to identify the exponential amplification phase of the reaction using a four-parameter logistic model. This algorithm is an objective and noise-resistant method to quantify qRT-PCR results. Expression levels of transcripts were normalized against two references genes, ribosomal protein L13a and 40 S ribosomal protein S30 FAU (Kokou et al., 2015) as well as also described for the European sea bass (Mitter et al., 2009). Normalization factors were calculated using geNorm (Vandesompele et al., 2002) as the geometric average of the two reference genes and following the instructions given by geNorm. In brief, after converting the C_t values into relative quantities using the delta C_t formula, gene expression levels were normalized by dividing the raw C_t values by the appropriate normalization factors. Expression levels and significance were confirmed by REST (Pfaffl, 2002). Outliers were identified using the online webtool at http://miniwebtool.com. Additionally the average mean of all possible triplets of the 24 expression values were calculated. For 24 values, 2024 possible triplets are obtained as the order is irrelevant and each value is presented only once in a triplet [(24!/21!*3!) = 2024].

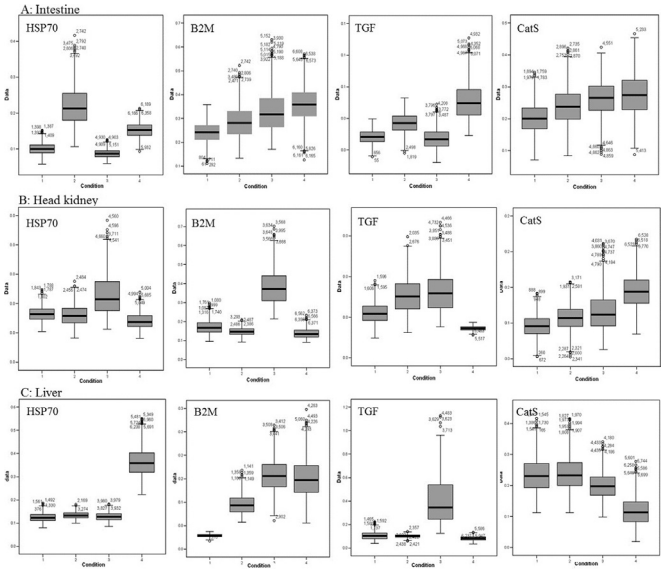

Fig. 1. Box-plot of expression averages values. Box-plot of average means of all possible triplets out of the 24 data points obtained from expression analysis.

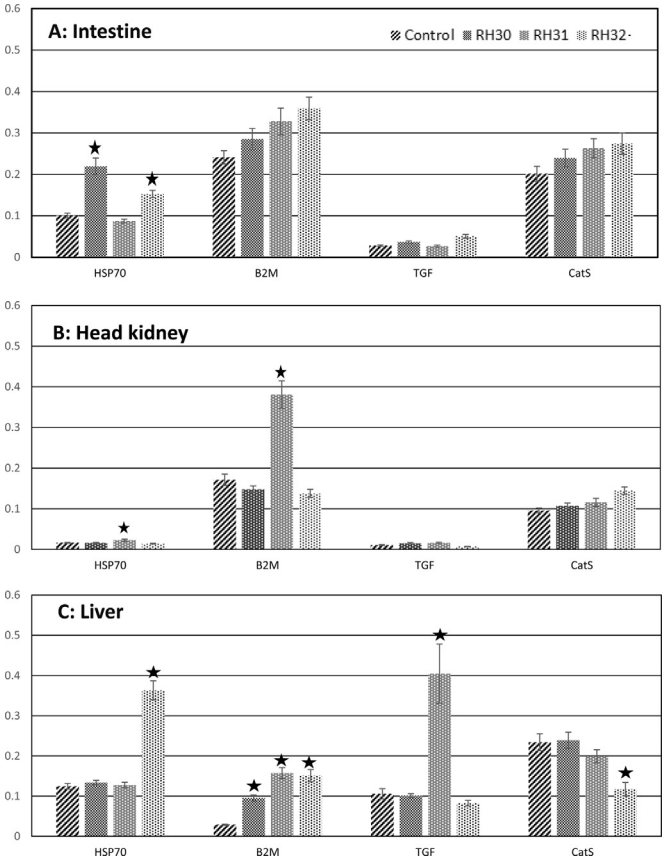

Fig. 2. Gene expression. Expression data for *hsp70, b2m, tgf, catS* in A: distal intestine, B: head kidney, C: liver. In liver also expression of IGF1 is shown.

3. Results and discussion

To ensure statistical significance in qPCR, the minimal information required comprises data about PCR efficiency, reference genes as well as sample size (Bustin, 2008). Most qPCR studies favor the use of internal references as normalization approach. However, in most of the studies due to additional costs only one reference gene is used (De Santis et al., 2011). In the present work two reference genes, *l13* and *fau* were used for normalization, which were also evaluated in precedent work (Kokou et al., 2015). Furthermore using a sample size of 24 and a confidence level of 95% encompass a confidence interval of 20. To investigate the possibility of using only three biological replicates, the average of all possible triplets were calculated and illustrated in Box-plot diagrams (Fig. 1). In the present study the significance of gene expression in all experiments when using 24 independent biological replicates were found for nine cases (Fig. 2). Looking at three biological replicates (Fig. 1) in only two cases, *hsp70* and *tgf* expression in liver, showed significant up-regulation regardless of the triplicate used for diets RH32 and RH31 respectively. On the other hand, looking e.g. at expression patterns of *hsp70* in intestine expression in RH30 differs significantly from the control and so is RH32 (Fig. 2a). Fig. 1a however shows that there are triplets that would not result in any differential expression especially concerning RH32. Even more obvious is the *hsp70* expression in head kidney for RH31 (Figs. Fig. 11 b and Fig. 22 b) whereas, as discussed before, the expression of *hsp70* in liver is robust whatever combination is taken and thus can be applied as trustable marker for immune response (Figs. Fig. 11 c and Fig. 22 c). The tendency of gene expression in all experiments remains the same, either when calculating all 24 independent biological replicates (Fig. 1) or when plotting all possible combinations of three (Fig. 2) showing the impact of the experimental set up, thus in the present work the impact of FM substitution. Taken together this case study shows the importance of choosing the adequate sample number, especially when differences are found to be significant but minor.

Looking at the different expression patterns based on the gradual decrease of FM in the dietary, apparently *hsp70* as well as *b2m* show a tissue specific expression pattern. *Hsp70* shows significant differences in the distal intestine for diets RH30 and RH32 (Fig. 2A) while in head kidney diet RH31 shows, a slight but statistical significant up-regulation to the control group for *hsp70* and a clear statistical significant up-regulation for *b2m* (Fig. 2B). In liver *hsp70* is noticeably up regulated only in RH32 whereas *b2m* shows in all three diets studied a statistical significant up-regulation in respect to the control diet. Heat shock proteins (Hsps) are known to be the key components in modulating stress responses (Sathiyaa and Vijayan, 2003). In fish *hsp70* has been shown to be involved in cytoprotection, cell survival and immune response (Basu et al., 2002; Aneja et al., 2006). Several studies have investigated the expression of immune related genes in gilthead sea bream fed with different diets (Calduch-Giner et al., 2013; Cerezuela et al., 2012; Montero et al., 2010; Reyes-Becerril et al., 2011a,b, 2008). In the present study the selection of genes (*hsp70*, *b2m*, *tgf* and *catS*) as well as target tissues (intestine, head kidney and liver) has been based on previous studies in cultured fish and mainly in salmonids. In Atlantic salmon e.g., Krogdahl et al. (2000) showed that diets containing an alcohol-extract of soybean meal had increased lysozyme activities and immunoglobulin in the intestinal mucosa. Head kidney macrophage activity was suppressed in rainbow trout at dietary inclusion levels of 60–70% of soybean products (Burrells et al., 1999). In liver studies regarding effects of fish meal substitution focused mostly on histological examinations. Steatosis for example was observed in the liver of gilthead sea bream juveniles after 100% substitution of FM by a plant protein mixture by Sitja-Bobadilla et al. (Sitjà-Bobadilla et al., 2005) and after 30% dietary inclusion of SBM by Robaina et al. (Robaina et al., 1995). Based on the results of the previous work in sea bream (Kokou et al., 2015) as well as in other species such as the Atlantic cod *Gadus morhua*, Atlantic salmon and Atlantic halibut *Hippoglossus hippoglossus* (Frøystad-Saugen et al., 2009; Hansen et al., 2006; Lilleeng et al., 2009; Murray et al., 2010; Olsen et al., 2007; Sagstad et al., 2007; Skugor et al., 2011) four genes with key roles in the immune response, i.e. *hsp70*, *b2m*, *tgf* and *catS* were selected for

qPCR analysis with the former two genes belonging to the MHCI pathway. In general *hsp70* is characterized by high inducibility throughout inflammation by an antagonizing effect of *hsp70*, representing an effort by the cell to avoid apoptosis (Calderwood et al., 2005). Beta 2 microglobulin (*b2m*) is an important subunit of major histocompatibility complex (*mhc*) type I molecules. Belonging to the genes involved in the key defense pathways, *b2m* has been shown to be up-regulated after infection e.g. (LeBlanc et al., 2010). In Atlantic halibut it was shown that genes involved in the immune system like *b2m* were up-regulated in the distal intestine after feeding with soybean meal. Up regulation of *b2m* in Atlantic halibut led to the hypothesis that the immune system may have been stimulated by the soybean meal diet or that pathogenic bacteria were increased due to a diseased gut.

Regarding the transforming growth factor beta (*tgf-β1*), a member of the gene family of cytokines, feeding experiments in the present study revealed statistical significant up-regulation of *tgf-β1* only in liver of RH31 fed fish. Several cytokines including *tgf-β1* have been isolated and characterized in fish and their mRNA expression level has been used for measuring immune response (e.g., Secombes et al., 2001). Contrary to the present work, studies in Atlantic salmon revealed a significant down regulation of *tgf-β1* in the distal intestine after the first day of soybean meal induction (Lilleeng et al., 2009). However no long-term studies are available and authors do not report about *tgf-β1* expression in liver. Modulatory effects of *tgf-β1* have been found on liver growth and repair in rats after hepatectomy (Russell et al., 1988). *Tgf-β1* expression has further been shown to correlate with continuing fibrotic injury in experimental animal models as well as in human liver diseases (Williams and Iredale, 2000) and is even considered as a suitable biomarker to detect liver lesion (Schon and Weiskirchen, 2014). In a previous study in the head kidney of sea bream we report up-regulation of *tgf-β1* at 40% substitution but not at 60%. In head kidney of sea bream it has also been shown, that the *tgf-β1* level is up regulated at 50 ng mL-1 of cortisol, corresponding to chronical stress and down regulated at 100 ng mL-1 which corresponds to the cortisol level found in acutely stressed sea bream (Castillo et al., 2008). These results may lead to the hypothesis that a lower substitution rate causes a response to the immune system whereas at high substitution level sea bream either is acutely stressed or it adapts to FM substitution.

Finally, Cathepsin S belonging to the lysosomal enzymes in particular to the lysosomal cysteine endopeptides of the papain family which are membrane-delimited organelles in animal cells were studied. Cathepsins are serving as the cell's key digestive compartment to which all sorts of macromolecules are delivered for degradation. Cathepsin S activity regulates antigen presentation and immunity (Riese et al., 1998). Additionally it has been shown that Cathepsin S is essential in early immune recognition (see Thurmond et al., 2005 for review). Interestingly, in the present study only for Cathepsin S in liver down-regulation was observed in individuals exposed to the RH32 diet. Cathepsin S, mediates invariant chain degradation of the mhcII/invariant chain complex in human and mouse in antigen-presenting cells in order to permit subsequent binding of peptides for generation of antigenic peptides. Down regulation of genes involved in the *mhcII* pathway i.e. gamma-interferon-inducible lysosomal thiol reductase (*gilt*) has been shown in the Atlantic salmon intestine fed with soy bean meal (Lilleeng et al., 2009). Authors showed down regulation of *gilt* which was suggested to be involved in the sensitization of intraepithelial lymphocytes (IEL) and which in turn results into the failure to regulate mucosal response towards antigens involving the secretion of suppressor cytokine *tgf-β1*. *tgf-β1* is up regulated in the present study in RH31 but not in RH32 which may be due to the down regulation of Cathepsin S. In the present study Cathepsin S did not show any other significant different expression.

4. Conclusion

The gradual substitution of fish meal by a plant mixture showed significant changes of the expression levels of four immune related genes. The present results revealed that only a lower substitution percentage (RH30) is tolerated and liver was proved to be the most sensitive tissue in terms of variance in gene expression. However gene expression was altered depending of the selected biological triplicate. This study showed that only for two genes in liver (*hsp70* and *tgf*) significant differential expression was assured independently of the triplicates used and thus can be applied as trustable marker for immune response using only three biological replicates as representatives. Concerning the genes studied, β2-M was the gene mostly affected by dietary treatment, and was found to be significantly up-regulated in head kidney and liver. In the distal intestine only *hsp70* was significantly up-regulated by PP inclusion, which was also observed in cod when fish meal was totally replaced by PP. Although the present results are robust due to the high number of biological replicates (24), it should be noted that they are still to be considered as first indications on the effect of PP inclusion in the diets for gilthead sea bream.

Conflicts of interest

The authors declare no conflict of interest.

Author contributions

E.S. conceived and designed the molecular experiments; F.K., J.K. and A.S. performed the fish nutrition experiments; F.K. carried out the molecular experiments, E.S. and F.K analyzed the data; A.S. and J.K. contributed reagents/materials/analysis tools; E.S, F.K., A.S and J.K. wrote the paper.

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